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Multiagent vaccines vectored by Venezuelan equine encephalitis virus replicon elicits immune responses to Marburg virus and protection against anthrax and botulinum neurotoxin in mice

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Abstract

The development of multiagent vaccines offers the advantage of eliciting protection against multiple diseases with minimal inoculations over a shorter time span. We report here the results of using formulations of individual Venezuelan equine encephalitis (VEE) virus replicon-vectored vaccines against a bacterial disease, anthrax; a viral disease, Marburg fever; and against a toxin-mediated disease, botulism. The individual VEE replicon particles (VRP) expressed mature 83-kDa protective antigen (MAT-PA) from *Bacillus anthracis*, the glycoprotein (GP) from Marburg virus (MBGV), or the H_C fragment from botulinum neurotoxin (BoNT H_C). CBA/J mice inoculated with a mixture of VRP expressing BoNT H_C serotype C (BoNT/C H_C) and MAT-PA were 80% protected from a *B. anthracis* (Sterne strain) challenge and then 100% protected from a sequential BoNT/C challenge. Swiss mice inoculated with individual VRP or with mixtures of VRP vaccines expressing BoNT H_C serotype A (BoNT/A H_C), MAT-PA, and MBGV-GP produced antibody responses specific to the corresponding replicon-expressed protein. Combination of the different VRP vaccines did not diminish the antibody responses measured for Swiss mice inoculated with formulations of two or three VRP vaccines as compared to mice that received only one VRP vaccine. Swiss mice inoculated with VRP expressing BoNT/A H_C alone or in combination with VRP expressing MAT-PA and MBGV GP, were completely protected from a BoNT/A challenge. These studies demonstrate the utility of combining individual VRP vaccines into multiagent formulations for eliciting protective immune responses to various types of diseases.

Keywords: Vaccine; Venezuelan equine encephalitis virus; VEEV; Botulinum neurotoxin; Anthrax; Marburg virus; Virus vector; VEE replicon; Multiagent vaccine

1. Introduction

Recent events involving *Bacillus anthracis* and increased concerns about the use of biological agents in acts of terrorism and warfare have increased the need for the rapid development of vaccines against a wide range of bacteria, toxins, and viruses [1]. The National Institute of Allergy and Infectious Diseases (NIAID) has classified biological organisms and

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14. ABSTRACT
The development of multiagent vaccines offers the advantage of eliciting protection against multiple diseases with minimal inoculations over a shorter time span. We report here the results of using formulations of individual Venezuelan equine encephalitis (VEE) virus replicon-vectored vaccines against a bacterial disease, anthrax; a viral disease, Marburg fever; and against a toxin-mediated disease, botulism. The individual VEE replicon particles (VRP) expressed mature 83-kDa protective antigen (MAT-PA) from Bacillus anthracis, the glycoprotein (GP) from Marburg virus (MBGV), or the H(C) fragment from botulinum neurotoxin (BoNT H(C)). CBA/J mice inoculated with a mixture of VRP expressing BoNT H(C) serotype C (BoNT/C H(C)) and MAT-PA were 80% protected from a B. anthracis (Sterne strain) challenge and then 100% protected from a sequential BoNT/C challenge. Swiss mice inoculated with individual VRP or with mixtures of VRP vaccines expressing BoNT H(C) serotype A (BoNT/A H(C)), MAT-PA, and MBGV-GP produced antibody responses specific to the corresponding replicon-expressed protein. Combination of the different VRP vaccines did not diminish the antibody responses measured for Swiss mice inoculated with formulations of two or three VRP vaccines as compared to mice that received only one VRP vaccine. Swiss mice inoculated with VRP expressing BoNT/A H(C) alone or in combination with VRP expressing MAT-PA and MBGV GP, were completely protected from a BoNT/A challenge. These studies demonstrate the utility of combining individual VRP vaccines into multiagent formulations for eliciting protective immune responses to various types of diseases.

15. SUBJECT TERMS
multiagent vaccine, VEE, replicon vaccine, botulinum neurotoxin, Bacillus anthracis, anthrax, filovirus, Marburg, efficacy, immune responses, laboratory animals, mice

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toxins that could be used in bioterrorism and biowarfare as category A, B, or C priority pathogens/agents. We have developed a multiagent vaccine utilizing the Venezuelan equine encephalitis (VEE) virus replicon as a vector for botulinum neurotoxin (BoNT), anthrax, and Marburg virus (MBGV), all of which are classified as category A pathogens/agents. The VEE vaccine vector system used in the studies described here is composed of an RNA replicon and a bipartite helper system for packaging the replicon into propagation-deficient VEE replicon particles (VRPs) [2]. The replicon contains a multiple cloning site immediately dow

nstream of the 26S promoter, which allows insertion of heterologous genes in place of the viral structural genes. Cotransfection (by electroporation) of cells in vitro with a recombinant VEE replicon and two helper RNA molecules, the latter encoding all of the VEE structural proteins, results in the production of propagation-deficient VRPs. When administered to an animal, the VRP infect host cells and lead to the production of immunogens that stimulate an immune response, but because the VRP lack structural genes, the infected cells do not produce progeny viral particles.

Anthrax is a disease caused by the sporulating bacterium *B. anthracis* and is usually associated with grazing animals. Humans may acquire the gastrointestinal or cutaneous forms of the disease through the handling or consumption of contaminated meat or from the processing of contaminated animal skins and wool [3]. Inhalational anthrax, the expected disease after a bioterrorism or biowarfare event, can result from the inhalation of spores and has an untreated case fatality rate of essentially 100%. The current human vaccine used in the United States, Anthrax Vaccine Absorbed (AVA), consists of an aluminum hydroxide-precipitated *B. anthracis* cell-free filtrate containing mostly protective antigen (PA), was licensed in 1970 by the Food and Drug Administration, requires a six-dose primary series and yearly boosters, and causes reactogenicity in up to 30% of vaccine recipients [3,4]. The ability of PA to elicit antibody responses and to protect animals from challenge has been demonstrated in a number of studies and that antibody responses in rabbits have been shown to correlate with protection [5–10]. In addition, anti-PA antibodies have been shown to function to prevent the effects of anthrax toxin [11,12], and also appear to inhibit spore germination and enhance the phagocytic and sporicidal activity of macrophages [12,13]. In anthrax vaccination studies using the VEE replicon, our group previously showed complete protection of A/J and CBA/J mice inoculated subcutaneously with VRP expressing the mature 83-kDa PA gene (MAT-PA) against a *B. anthracis* (Sterne strain) challenge [14].

Clostridium botulinum, the organism that causes botulism, produces one or more of the seven serotypes of BoNT (serotypes A–G). The disease resulting from the activity of BoNT occurs through the inhibition of neuromuscular stimuli [15–17]. Food poisoning, infant botulism, and wound botulism are the three most common BoNT diseases affecting humans. BoNTs are the most toxic compounds known, with an estimated toxic dose of 1 ng/kg of body weight. Previous research showed that polyclonal antibodies to one serotype can only block the effects of the homologous serotype [18]. The current human vaccine, which is administered under Investigational New Drug (IND) status to at-risk laboratory personnel, is prepared as a toxoid and contains five of the seven serotypes (A–E). The toxoid vaccine is given as a primary series of four inoculations administered at 0, 2, 12, and 24 weeks, followed by a booster at 1 year, and is reactogenic in up to 20% of the recipients. The vaccine is expensive to manufacture and producing the large amounts of active toxin necessary for the toxoiding process poses safety and security issues. Vaccines composed of recombinant BoNT fragment C (H_C) combined with adjuvant have been shown to protect mice from the effects of homologous toxin challenges and that antibody responses correlated with protection [19–21]. Vaccination of humans with toxoided BoNT F combined with adjuvant has been shown to stimulated antibody responses that were determined to be protective in a mouse bioassay [22]. We previously cloned the BoNT/A H_C fragment into the VEE replicon and have shown complete protection in mice inoculated with the corresponding VRP vaccine (A/H_C VRP) [23]. The mice produced high antibody responses, survived challenges of up to 100,000 LD_{50} units of BoNT/A, and were protected for up to 1-year post-vaccination.

Marburg virus (MBGV), a filovirus belonging to the *Filoviridae* family, has the potential for aerosol dissemination and weaponization by terrorists. Originally recognized in 1967, MBGV has caused few natural outbreaks and remains as a sporadic disease in southeast Africa [24,25]. Several methods have been used to develop MBGV vaccines and include using DNA expressing glycoprotein (GP) [26], irradiated (killed) MBGV [27], or baculovirus-expressed truncated GP [27]. Gene-gun inoculations of approximately 2.5 μg of DNA divided between four sites on the abdomens of guinea pigs protected 100% of the animals from a lethal challenge of MBGV. Previous results by others showed that guinea pigs inoculated with VRP expressing MBGV GP or nucleoprotein (NP) were completely protected from a lethal MBGV challenge [28]. Because of safety concerns, cloned genes from MBGV are more desirable than using irradiated MBGV in the development of multiagent vaccines. In the studies presented here, we demonstrated immunogenicity against MBGV and efficacy against anthrax and botulinum neurotoxin of multiagent VEE replicon-vectored vaccines in mice.

2. Materials and methods

2.1. Preparation of the VRP vaccines

Construction of the VEE replicon vector, the capsid helper, and the glycoprotein helper, which contains attenuating mutations, was as previously described [2]. Construction, characterization, and assembly of the replicons into VRP for the BoNT/A H_C, the anthrax MAT-PA replicon, the MBGV-GP

replicon, and the negative control comprising mutagenized staphylococcal enterotoxin B (mSEB) replicon were prepared as previously published [14,23,28,29]. The BoNT/C H_C gene was PCR-cloned into the VEE replicon plasmid by using Cla I restriction enzyme recognition sequence—gene specific primers. The VRP titers are expressed as focus-forming units (FFU) where 1 FFU is equivalent to 1 infectious unit (iu).

2.2. Vaccination and challenge of mice

Swiss and CBA/J mice were inoculated subcutaneously (s.c.) behind the neck with either a single VRP or with a mixture of VRPs at a dose of 10^7 iu of each VRP in 200 μl of phosphate-buffered saline (PBS) on days 0, 35, and 70. No adverse reactions or side effects were noted in mice receiving individual or combination VRP vaccines. The mice were challenged intraperitoneally (i.p.) on day 105 or 164 with 1000 50% median lethal doses (MLD_{50}) of BoNT/A or BoNT/C (Metabiologics, Inc., Madison, WI) diluted in PBS containing 0.2% gelatin, respectively. CBA/J mice were challenged s.c. on day 105 with 10 LD_{50} units (2×10^8 spores) of heat-shocked spores of the Sterne strain of *B. anthracis*. Positive control mice were inoculated s.c. with 0.1 ml of anthrax vaccine adsorbed (AVA, Bioport Corp., Lansing, MI) or with 0.1 ml of pentavalent botulinum toxoid vaccine (serotypes ABCDE, Bioport Corp., Lansing, MI), and negative control mice were inoculated with mSEB VRP, and all were used as controls for the challenges. As a comparison, the dose of AVA or toxoid vaccine administered to humans is 0.5 ml. Sera for ELISA were obtained 2 days before each inoculation and 2 days before challenge.

2.3. Serum ELISA

The quantity of total IgG antibody present in the serum of vaccinated animals was measured by ELISA as previously described [14,30,31]. Briefly, microtiter plates were coated with either *E. coli*-expressed PA protein (1 μg/ml), purified *C. botulinum* expressed BoNT/A or BoNT/C protein (1 μg/ml), or with purified, irradiated MBGV (2 μg/ml total protein) diluted in PBS. Titers for BoNT/A, BoNT/C, and PA, or for MBGV are defined as the reciprocal of the last dilution with an A_{405} of ≥0.1 or A_{405} of ≥0.2, respectively, after correction for background. Titers below 2.00 $\log_{10}$ and above 5.61 $\log_{10}$ were estimated for BoNT/A, BoNT/C, and PA and below 1.5 $\log_{10}$ and above 5 $\log_{10}$ were estimated for MBGV. Serum from individual animals was assayed in duplicate and used to calculate a geometric mean titer for the group.

2.4. Statistical analysis

Using a one-tailed Fisher's exact test to compare survival rates between the control and treatment groups, the experiments described here require a sample size of 10 mice per group for adequate (≥90%) power. This sample size would allow the experimenter to detect a minimum efficacy rate of 60% (6/10 surviving) in the treatment group compared to 0% (0/10 surviving) in the control group at a 95% confidence level.

Analyses were performed on $\log_{10}$-transformed reciprocal dilutions of ELISA titers. After transformation, ELISA titers met assumptions of normality and homogeneity of variance. *T*-tests were used to compare titers between specific groups, with stepdown Bonferroni correction used for multiple comparisons. Fisher exact tests were used to compare survival rates between specific groups, also with stepdown Bonferroni correction used for multiple comparisons. Analyses were conducted using SAS Version 9.1 (SAS Institute, Inc., SAS OnlineDoc, Version 9.1, Cary, NC, 2004).

3. Results

3.1. Immunogenicity of individual VRP vaccines as compared to mixtures of VRP vaccines

Table 1 shows the antibody responses for Swiss mice inoculated with individual VRP or with mixtures of VRP vaccines. Swiss mice inoculated on days 0, 35, and 70 with 10^7 iu of an individual BoNT/A H_C VRP produced an antibody response of 5.68 $\log_{10}$ against BoNT/A, as measured in serum collected on day 103, 2 days before challenge. Inoculation of Swiss mice with the same dose of BoNT/A H_C VRP but in combination with either MAT-PA VRP or MBGV-GP VRP elicited similar antibody titers of less than two-fold difference, 5.79 and 5.90 $\log_{10}$, respectively ($p > 0.05$). Additionally, a six-fold increase in antibody titer against BoNT/A H_C, 6.44 $\log_{10}$, was noted for mice receiving a formulation of all three VRP vaccines ($p < 0.05$).

Comparing antibody responses produced by Swiss mice inoculated with MAT-PA VRP or in combination with the other VRP vaccines showed results similar to those noted above for Swiss mice inoculated with individual or mixed BoNT/A H_C VRP vaccines. Mice inoculated with MAT-PA or MAT-PA and BoNT/A H_C VRP produced the same antibody titer of 4.53 $\log_{10}$ against PA protein as measured on day 103 as compared to mice inoculated with MAT-PA combined with MBGV-GP VRP that produced an approximately four-fold higher antibody response of 5.13 $\log_{10}$ ($p = 0.05$). The combination of all three VRP vaccines stimulated antibody responses in the mice that were intermediate with a titer of 4.92 $\log_{10}$ against PA protein which was approximately two-fold higher than the antibody response stimulated by MAT-PA VRP only or approximately two-fold lower than the response stimulated by the MAT-PA VRP plus MBGV-GP VRP mix.

Swiss mice inoculated with MBGV-GP VRP, either as an individual vaccine or in combination with the other VRP vaccines, produced similar antibody responses that varied approximately two-fold or less ($p > 0.05$). MBGV-GP VRP stimulated an antibody response in the mice of 4.68 $\log_{10}$

Table 1
Antibody responses and protection in Swiss mice inoculated with multiagent VRP vaccines and challenged with BoNT/A

Inoculum[a]	Dose (iu or ml)[b]	Geometric mean titer, $\log_{10}$ (S.D.)[c]			Challenge agent[d]	Survived/total[e]
		BoNT/A	PA	MBGV GP		
BoNT toxoid vaccine	0.1 ml	6.08 (0.25)			BoNT/A	9/9
Anthrax Vaccine Absorbed (AVA)	0.1 ml		6.39 (0.34)			
BoNT/A Hc VRP	10^7	5.68 (0.35)		2.86 (0.24)	BoNT/A	9/9
MAT-PA VRP	10^7		4.53 (0.54)			
MBGV-GP VRP	10^7	2.36 (0.57)	2.72 (0.25)	4.68 (0.24)	BoNT/A	0/10
BoNT/A Hc VRP + MAT-PA VRP	10^7 each	5.79 (0.28)	4.53 (0.72)		BoNT/A	10/10
BoNT/A Hc VRP + MBGV-GP VRP	10^7 each	5.90 (0.65)		4.53 (0.41)	BoNT/A	10/10
MAT-PA VRP + MBGV-GP VRP	10^7 each		5.13 (0.54)	4.35 (0.46)		
BoNT/A Hc VRP + MAT-PA VRP + MBGV-GP VRP	10^7 each	6.44 (0.61)	4.92 (0.66)	4.41 (0.46)	BoNT/A	19/19

[a] Mice were inoculated s.c. on days 0, 35, and 70 with the indicated vaccines.
[b] Infectious units were used to measure VRP and milliliters were used to measure toxoid and AVA inocula.
[c] The indicated proteins were used to coat ELISA plates and serum was obtained from animals bled on day 103.
[d] Animals were challenge i.p. on day 105 with 1000 LD_{50} units of BoNT/A.
[e] Significant survival was noted for all groups as compared to the appropriate negative control, $p < 0.05$.

against purified MBGV as measured on day 103 as compared to antibody responses of 4.53, 4.35, and 4.41 $\log_{10}$ for mice inoculated with MBGV-GP VRP combined with BoNT/A H_C, MAT-PA, or BoNT/A H_C and MAT-PA VRP, respectively, with no statistical differences noted between the responses ($p > 0.05$). In general, Swiss mice receiving mixes of VRP vaccines containing BoNT/A H_C or MAT-PA produced higher (BoNT/A H_C or MAT-PA specific) antibody titers than those receiving the individual vaccines while mice that received mixes of VRP vaccines containing MBGV-GP VRP or the individual vaccine produced comparable MBGV-GP specific antibody titers.

Additional experiments in a second strain of mice, CBA/J, produced results similar to those observed for the Swiss mice except that the mixed VRP vaccines stimulated lower antibody responses as compared to the individual VRP vaccines (Table 2). Inoculating CBA/J mice with BoNT/A H_C VRP stimulated approximately three-fold less serum anti-

Table 2
Antibody responses and survival of CBA/J mice inoculated with different VRP vaccines and then challenged with BoNT/A, BoNT/C, or with *B. anthracis* (Sterne strain)

Inoculum[a]	Dose (iu or ml)[b]	Geometric mean titer, $\log_{10}$ (S.D.)[c]			First challenge agent[d]	First challenge survived/total	Second challenge agent[e]	Second challenge survived/total
		BoNT/A	PA	BoNT/C				
BoNT toxoid vaccine	0.1 ml	6.48 (0.22)		4.53 (0.61)	BoNT/A	10/10[f]	BoNT/C	9/10[f]
Negative control VRP	10^7	1.80 (0.41)			BoNT/A	0/10	nc	
Anthrax Vaccine Absorbed (AVA)	0.1 ml	2.02 (0.70)	6.12 (0.29)		*B. anthracis* Sterne	10/10[f]	BoNT/A	0/10
Negative control VRP	10^7		1.69 (0.54)		*B. anthracis* Sterne	2/10	nc	
BoNT/A Hc VRP	10^7	5.22 (0.79)			BoNT/A	9/10[f]	BoNT/A	9/9[f]
MAT-PA VRP	10^7		5.16 (0.73)	1.33 (0.53)	*B. anthracis* Sterne	10/10[f]	BoNT/C	0/10
BoNT/A Hc VRP + MAT-PA VRP	10^7 each	4.89 (0.91)	4.89 (0.86)		BoNT/A	2/10	nc	
BoNT/A Hc VRP + MAT-PA VRP	10^7 each	4.53 (1.29)	4.65 (1.08)		*B. anthracis* Sterne	9/10[f]	BoNT/A	4/9[f]
BoNT/C Hc VRP + MAT-PA VRP	10^7 each		4.14 (1.35)	2.84 (0.30)			BoNT/C	10/10[f]
BoNT/C Hc VRP + MAT-PA VRP	10^7 each		5.19 (0.73)	3.14 (0.19)	*B. anthracis* Sterne	8/10[f]	BoNT/C	8/8[f]

[a] Mice were inoculated s.c. on days 0, 35, and 70 with the indicated vaccines. Negative control groups were inoculated with an unrelated VRP.
[b] Infectious units were used to measure VRP and milliliters were used to measure toxoid and AVA inocula.
[c] The indicated proteins were used to coat ELISA plates and serum was obtained from animals bled on day 103.
[d] Animals were challenged s.c. on day 105 with either 10 LD_{50} units (2×10^8 spores) of *B. anthracis* (Sterne) or with 1000 MLD_{50} units of BoNT/A as indicated.
[e] Animals were successively challenged i.p. on day 164 with either 1000 LD_{50} units of BoNT/A or with 1000 MLD_{50} units of BoNT/C as indicated.
[f] Significant survival was noted for these groups as compared to the appropriate negative control, $p < 0.05$.

bodies as measured by ELISA, 5.22 $\log_{10}$ against BoNT/A, as compared to antibody levels of 5.68 $\log_{10}$ for the Swiss mice inoculated with the same vaccine ($p > 0.05$). CBA/J mice produced approximately 12-fold less antibody when inoculated with a mix of BoNT/A H_C and MAT-PA VRP, 4.89 $\log_{10}$ for one group and 4.53 $\log_{10}$ for the second group against BoNT/A, respectively, as compared to the antibody levels of 5.79 $\log_{10}$ for the Swiss mice that received the same combination of vaccines ($p < 0.05$). The antibody response stimulated in CBA/J mice inoculated with MAT-PA VRP, 5.16 $\log_{10}$ against PA protein, was approximately four-fold higher than the antibody response measured for the Swiss mice, 4.53 $\log_{10}$, that received the same vaccine ($p < 0.05$). The antibody responses for CBA/J mice that received mixes of MAT-PA VRP and BoNT/A H_C, 4.89 $\log_{10}$ for one group and 4.65 $\log_{10}$ for a second group against PA protein, was approximately three-fold lower than the antibody response measured for the corresponding mice that received only MAT-PA VRP, 5.16 $\log_{10}$, and was either approximately two-fold higher or about the same as the response of 4.53 $\log_{10}$ measured for Swiss mice inoculated with the same two VRP vaccines, MAT-PA VRP and BoNT/A H_C, respectively. Overall, the levels of antibody present in the CBA/J mice against BoNT/A trended lower than those determined for the Swiss mice, yet remained about the same against PA for both mouse strains.

3.2. *Efficacy of individual VRP vaccine as compared to mixtures of VRP vaccines.*

Inoculation of Swiss mice on days 0, 35, and 70 with 10^7 iu of BoNT/A H_C VRP, either alone or in combinations with MAT-PA or MBGV-GP, or with all three VRP vaccines, completely protected the mice for challenge with BoNT/A (Table 1). As expected, Swiss mice that only received MBGV-GP VRP were not protected against a BoNT/A challenge. Because Swiss mice are not affected by MBGV or the Sterne strain of *B. anthracis*, we were unable to challenge the mice with these agents.

Challenge of CBA/J mice with the Sterne strain of *B. anthracis* did affect these mice and allowed for efficacy testing of the vaccines containing MAT-PA VRP (Table 2). CBA/J mice inoculated with MAT-PA VRP or with a mix of BoNT/A H_C and MAT-PA VRP were either completely or 90% protected from challenge with Sterne, respectively. Successive BoNT/A challenge of CBA/J mice that had previously received a mix of BoNT/A H_C and MAT-PA VRP and that had survived the Sterne challenge on day 105, demonstrated that four of nine mice (44%) were protected on day 164 from the effects of BoNT/A.

CBA/J mice inoculated with BoNT/A H_C VRP were 90% protected from a BoNT/A challenge whereas a mix of BoNT/A H_C and MAT-PA VRP poorly protected the animals (2 of 10 mice survived) from the same BoNT/A challenge. In a comparison study, CBA/J mice inoculated a mixture of C/H_C and MAT-PA VRP were completely protected from a BoNT/C challenge or were 80% protected from a Sterne challenge. In the successive challenge experiment, the eight mice that survived the Sterne challenge were also completely protected from a BoNT/C challenge.

4. Discussion

The development of vaccines that require fewer inoculations and that protect against multiple diseases or agents will benefit first responders and at-risk laboratory personnel. Here we report the use of the VEE replicon vector system in the formulation of multiagent vaccines against BoNT, MBGV, and *B. anthracis*. Previous studies established the immunogenicity and efficacy of the individual VEE replicon-vectored vaccines, thus enabling the formulation of the multiagent vaccines reported here [14,23,28]. Swiss mice responded well to each of the components in the multiagent vaccine, as measured by serum ELISA titers, and were completely protected from a BoNT/A challenge. Increased antibody responses against BoNT/A, approximately six-fold, were measured for Swiss mice inoculated with the three VRP vaccines as compared to mice inoculated with the individual BoNT/A H_C VRP vaccine ($p < 0.05$). In contrast, antibody responses to the other immunogens varied by less than two-fold ($p > 0.05$) between the animals inoculated with the individual VRP vaccine as compared to animals inoculated with the mixed VRP vaccines, except that the antibody response increased by four-fold in the Swiss mice that received the MAT-PA–MBGV-GP VRP mix as compared to those mice that received only MAT-PA VRP ($p < 0.05$). This demonstrates that the individual VRP components in the multiagent vaccine formulation do not interfere and that it is possible to create other multiagent vaccines using the VEE replicon.

Since Swiss mice are not responsive to MBGV or *B. anthracis* (Sterne strain) challenge, and that a *B. anthracis* (Ames strain) challenge can kill mice independent of their vaccination status [32], a second mouse strain, CBA/J, was evaluated in a preliminary study to investigate the efficacy of mixing BoNT/A H_C or C/H_C VRP with the MAT-PA VRP vaccine. CBA/J mice inoculated with a mix of BoNT/A H_C and MAT-PA VRP were poorly protected (2 of 10 mice survived, $p > 0.05$ as compared to the negative control VRP group) from a BoNT/A challenge as compared to 90% protection in the same stain that received only BoNT/A H_C VRP ($p < 0.05$). In contrast, a different group of CBA/J mice inoculated with the same mix of BoNT/A H_C and MAT-PA VRP vaccine were 90% protected from a Sterne strain challenge ($p < 0.05$) and that four of those nine surviving mice were also protected against a BoNT/A challenge ($p < 0.05$ as compared to the negative control VRP group). From these data, one could conclude that the MAT-PA VRP vaccine is equally immunogenic in Swiss mice as it is in CBA/J mice, as the ELISA titers were similar between the two mouse strains, and that BoNT/A H_C is less immunogenic in CBA/J mice than it is in Swiss mice. The ELISA titers

for the Swiss mice that received a mix of the BoNT/A H_C and MAT-PA VRP vaccines were on average 12-fold higher than the corresponding CBA/J groups ($p < 0.05$). This large difference in titers explains why the mice were not protected and may have resulted from a diminished immunogenicity of the BoNT/A H_C immunogen in the mice. For example, the inbred CBA/J mice, as compared to the outbred Swiss mice, may have had a limited ability to recognize important epitopes in BoNT/A involved in B-cell stimulation or in $CD4^+$ helper T cell activation or that interference caused by the MAT-PA VRP may have prevented proper expression of BoNT/A H_C from the VEE replicon in some animals.

In this study, CBA/J mice inoculated with either BoNT/A H_C or C/H_C and MAT-PA VRP were evaluated for their ability to respond to the mixed VRP vaccines. The antibody responses against PA for each of the two groups of CBA/J mice inoculated with BoNT/A H_C or C/H_C VRP mixed with MAT-PA VRP (for a total of four groups) was 4.89 and 4.65 $\log_{10}$ or 4.14 and 5.19 $\log_{10}$, respectively, was similar to the antibody response of 4.53 $\log_{10}$ measured for the Swiss mice inoculated with the same BoNT/A H_C and MAT-PA VRP combination (no significant differences, $p > 0.05$). Even though a group of CBA/J mice inoculated with only C/H_C VRP was not included in the experiment, challenge of the CBA/J mice inoculated with C/H_C and MAT-PA VRP showed that the combination vaccine could protect 100% of the mice from a BoNT/C challenge. The successive challenge experiment showed that CBA/J mice previously challenged with Sterne were also 100% protected from a BoNT/C challenge. The antibody response to the C/H_C component of the combination vaccine was considerably less than that measured for the BoNT/A H_C component in the CBA/J mice that had received the BoNT/A HC–MAT-PA VRP combination. Even though the titers differed by more than 50-fold, the antibody response protected all the CBA/J mice from a BoNT/C challenge. These data indicate that the quality, i.e., neutralizing verses non-neutralizing antibody activity, of the anti-C/H_C antibody response was considerably better than the anti-A/H_C antibody response measured for CBA/J mice or that the BoNT/C circulated in the animals for longer periods before entering neurons thus allowing more time for antibody-mediated neutralization of the toxin in the animal after challenge. Additional experiments involving the use of higher doses of BoNT/A H_C VRP combined with the same amount of MAT-PA VRP may help determine the parameters necessary to protect CBA/J mice from the effects of BoNT/A. Also vaccinating additional strains of mice with the BoNT/A H_C–MAT-PA VRP combination vaccine may help to determine if the poor antibody responses to BoNT/A H_C, when it was included in mixes with MAT-PA VRP, are universal or if they are mouse-strain specific.

The advantages of formulating multiagent vaccines by using the VEE replicon vector system were demonstrated by these studies. Specifically, individual vaccine components, i.e., the different VRPs, can be easily combined without complicated mixing requirements, mixed VRP vaccines are as immunogenic as the individual VRP vaccines, and formulations of VRP vaccines provide similar levels of protection as compared to individual VRP vaccines. Other published studies have shown the effectiveness of multiagent vaccines based on DNA; however, those studies relied on separate administration of each of the vaccine components [26]. The study reported a total of 24 inoculations split between three vaccination periods. Our multiagent VRP vaccine only required one inoculation per vaccination period thus making this type of vaccine versatile, easily administered, and more acceptable to the recipient. Multiagent vaccine formulations using the VEE replicon as a vector to express immunogens in a host have broad applications for both public health and for biodefense. The multiagent vaccine reported here stimulated antibody responses to three NIAID category A pathogens and provided near complete protection against *B. anthracis* (Sterne strain) and provided complete protection against BoNT. Continued development of multiagent vaccines, either as DNA, protein plus adjuvant, VEE replicon, or other virus vectors, is necessary for creating user-friendly vaccines that protect against the long list of public health and biological threat agents present in the world today.

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